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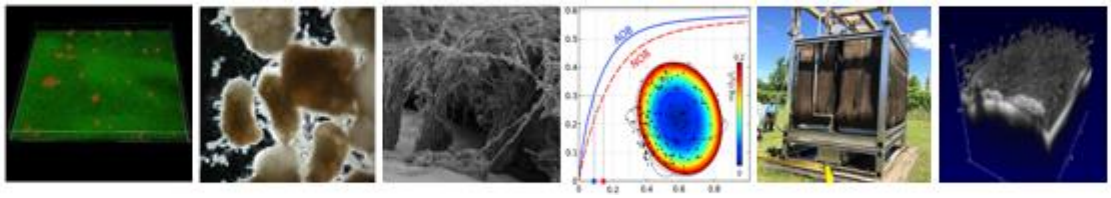

# Impact of substrate concentration on granular fermentation for caproic acid production

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## Summary of key findings

Recently, our laboratory demonstrated the feasibility of high rate caproic acid production, up to 13.7 $g \cdot L^{-1} \cdot d^{-1}$ from a biorefinery sidestream, by chain elongating granular biofilms in an expanded granular sludge bed reactor. In this study, we investigated the effect of substrate concentration on this system as a crucial first step towards feedstock diversification. We observed improved aggregation and granulation with decreasing substrate concentrations, leading to higher retained biomass concentrations. Conversely, higher substrate concentrations led to larger granules. Additionally, we found that caproic acid product selectivity remained constant at 48 ± 3% with increasing substrate concentration, up until 50 $gCOD \cdot L^{-1}$, after which product toxicity exerted by high caproic acid concentrations shifted the selectivity towards butyric acid.

## Background and relevance

Today's increasing organic waste production poses a challenge for the conversion of liquid waste streams into added-value products beyond low-value methane. A suitable alternative has been suggested to be the production of medium chain carboxylic acids (MCCAs) via a microbial process called chain elongation. In this process, an electron donor – e.g. ethanol or lactic acid– is used to elongate acetic acid to butyric acid, which is in turn elongated to caproic acid. MCCAs, and in particular caproic acid (C6), have a multitude of applications as feed additives, antimicrobial compounds and can be utilised in production of fragrances, rubbers, dyes and pharmaceuticals (Angenent et al., 2016). Lactic acid chain elongation has been proposed as a suitable alternative to ethanol, due to its potential coupling with lactic acid production from carbohydrates, eliminating the need for exogeneous ethanol addition (Chen et al., 2017; Zhu et al., 2015).

One key limitation in these production systems are production rates, an issue that was raised early on in the new wave of MCCA-research of the 2010s (Agler et al., 2011). Granular sludge are self-aggregating biofilms, offering retention of high biomass concentrations that in turn enable higher conversion rates. These have been applied in other wastewater treatment technologies, and have recently found their way to MCCA production (Carvajal-Arroyo et al., 2019; Roghair et al., 2016; Wu et al., 2021). Moreover, our group recently demonstrated the feasibility of C6 production using fermentative granular biofilms in an expanded granular sludge bed (EGSB) reactor, by achieving productivities up to 13.7 $gC6 \cdot L^{-1} \cdot d^{-1}$ from a real biorefinery sidestream (Carvajal-Arroyo et al., 2019). However, the potential of this system was only demonstrated with a single feedstock and little is known about its benefits for other differently concentrated streams such as wastewaters from the brewery, dairy and food processing industries. Here, we investigated the effect of substrate concentration as a first step towards feedstock diversification for high rate granular C6 production.

## Results

Reduced substrate concentrations were investigated by diluting solids-free thin stillage (total substrate concentration of 44.15 $gCOD \cdot L^{-1}$; total carbohydrate content of 19.76 $g \cdot L^{-1}$), to respectively 75%, 50% and 25% of its substrate concentration and feeding it to an EGSB reactor. In terms of product spectrum, the C6 concentration in the effluent decreased from 4.35 ± 0.25 $g \cdot L^{-1}$ at 100% to 1.59 ± 0.27 $g \cdot L^{-1}$ at 25%. However, the overall C6 selectivty remained constant at 48 ± 3% and lower substrate concentrations resulted in higher conversion efficiencies (Figure 1.1). Lower substrate concentrations also consistently yielded the highest amounts of total biomass (combination of planktonic and granular) due to substantial growth of the granular bed (Figure 2.1). Illumina 16S rRNA sequencing revealed that the reactor community was largely dominated by OTU classified as *Caproiciproducens* (86.41% of granular and 79.11% of planktonic community) and the lactic acid producing *Olsenella* genus (7.3% of granular and 17.68% of planktonic community). Below 44.15 $gCOD \cdot L^{-1}$, granule size was not affected, with granular diameters between 2.3 and 3.7 mm. Noteworthy was the appearance of floccular biomass at 25% stillage feedstock (Figure 2.1).

In a second experiment, solids-free thin stillage was amended with D-glucose to respectively 110%, 125% and 200% of the original COD. At increased carbohydrate loading, butyric acid accumulated, eventually reaching up to similar concentrations as C6 (from 1.78 ± 0.20 $g \cdot L^{-1}$ at 100% to 4.03 ± 0.48 $g \cdot L^{-1}$ at 125%; C6 at 4.35 ± 0.49 $g \cdot L^{-1}$; Figure 1.1). This in turn resulted in a strong decrease in C6 product selectivity. Analysing granule size at increased carbohydrate loading was not possible due to granule instability during sampling, but granules were visually larger (diameter approximately up to 1 cm; Figure 2.1).

**Discussion**

In this study, we investigated the response of C6 producing granular biofilms to varying substrate concentrations as a proxy for its potential to convert different waste feedstocks, leading to three key observations: (i) reduced substrate concentrations do not affect selectivity and increase conversion efficiency, (ii) increased substrate concentrations reduce selectivity and reduce conversion efficiency, and (iii) increased substrate concentrations affect granule growth and size although mechanisms are unclear.

The constant selectivity and increased substrate conversion efficiencies at low substrate concentrations demonstrate that high COD concentrations are no prerequisite for C6 production in an EGSB. However, the low product concentrations may pose challenges for efficient product extraction. High substrate concentrations on the other hand do not lead to increased C6 concentrations, likely due to product toxicity. MCCAs exert toxicity due to their amphipathic structure, which allows insertion of the undissociated acids in the membrane and even migration followed by acidification of the cytoplasm. Planktonic ethanol chain elongation microbiomes have been reported to be inhibited at undissociated C6 concentrations around 7.5 mM, which are comparable to those achieved in this study of 7.25 ± 0.85 mM (Ge et al., 2015). Other planktonic lactic acid chain elongation studies have achieved undissociated C6 concentrations ranging from 7.4 mM (Zhu et al., 2015) up to 17.2 mM (Duber et al., 2018). The use of a granular biofilm may impose additional diffusional limitations and increase product toxicity since high intra-granule C6 concentrations may limit further conversion and lead to lower bulk C6 concentrations. Overall, this implies in-situ product extraction may be necessary to maintain high selectivities.

The consistent observation of highest amounts of total biomass at lower substrate concentrations suggest that substrate limitation and/or starvation may have a positive impact on biomass aggregation and thus retention in the reactor, but the mechanism remains unclear. Granules in the 25% to 100% periods were of similar size to those found by Carvajal-arroyo et al., but substantially larger than other fermentative granules earlier reported (Roghair et al., 2016; Tamis et al., 2015; Wu et al., 2021). The larger granules observed at higher substrate concentrations have not been reported before and their formation mechanism remains unclear.

Overall, this study demonstrates that chain elongation in EGSBs offers a strong tool for the valorisation of carbohydrate-rich streams and could be expanded towards more dilute and concentrated waste streams in combination with efficient in-situ product extraction.

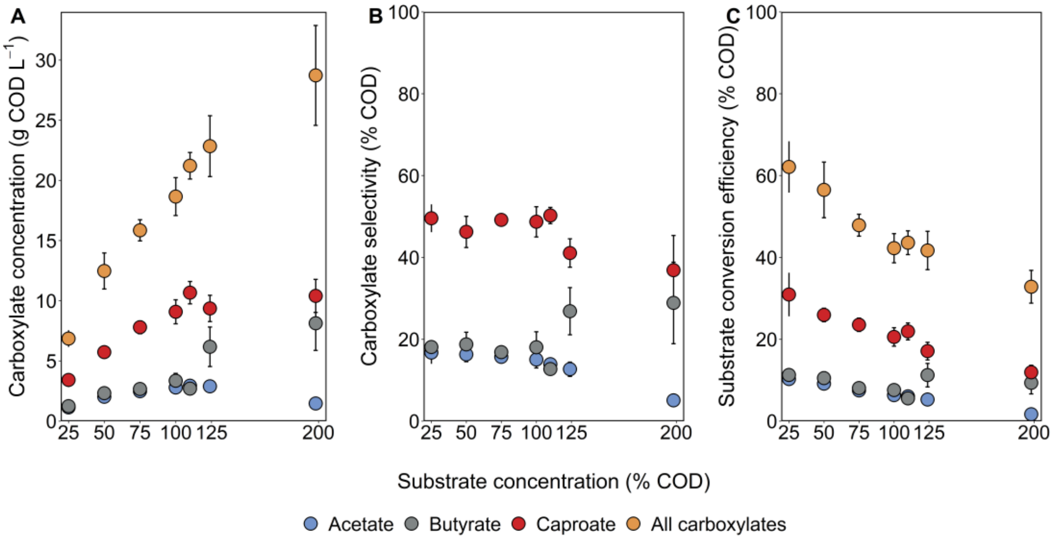


**Figure 1.1 Carboxylic acid concentration (A), selectivity (B) and substrate conversion efficiency (C) in function of the substrate concentration.**

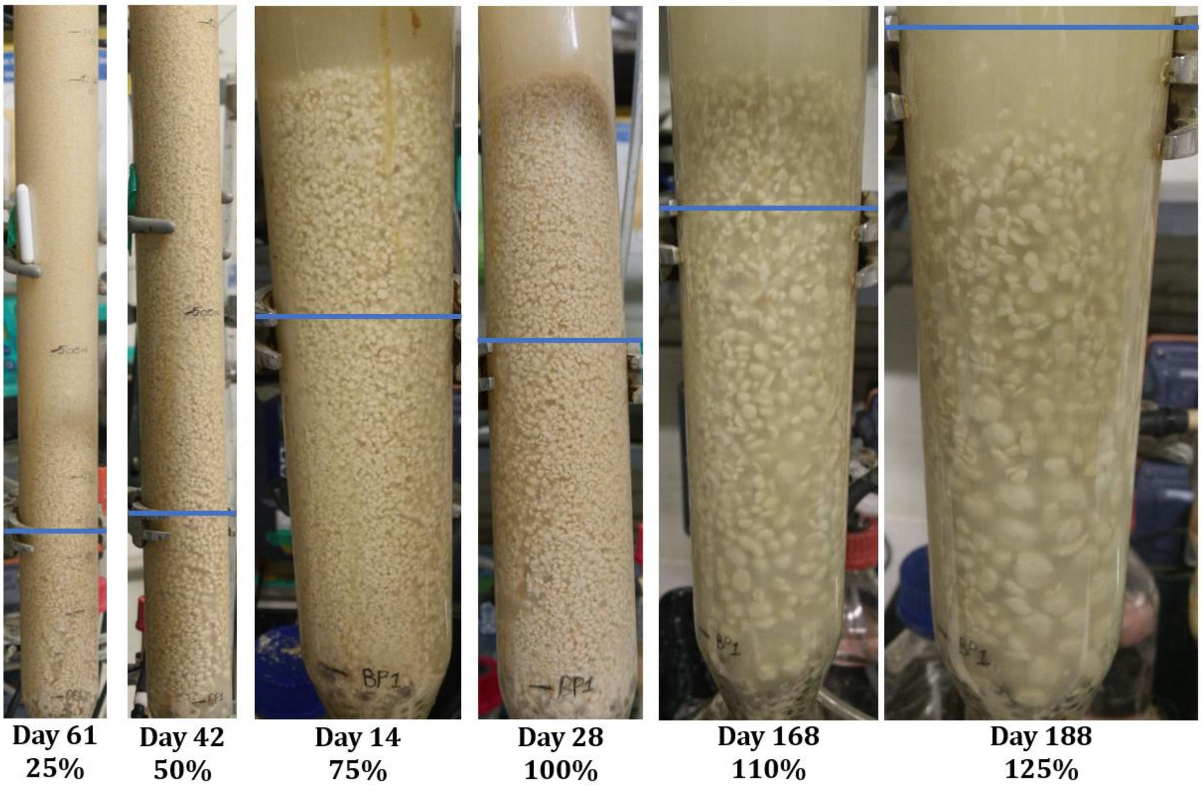


**Figure 2.1 Evolution of the granular bed throughout varying substrate concentrations. The blue line indicates the same reference height in each picture.**

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**Is the presenting author an IWA Young Water Professional? ~~Y~~/N**
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**Bio:** Quinten Mariën is a bioprocess engineer and PhD researcher. After graduating with a Master in bioscience engineering, specialised in chemistry and bioprocess technology, he obtained a PhD fellowship from the Flanders Research Foundation. He is fascinated by the potential of microbial technology in resource recovery and valorization from liquid and gaseous waste streams. His PhD research at Ghent University (Belgium) focuses on the development of novel biofilm reactor technologies for high-rate fermentation and carbon capture. In his off time, he enjoys nature, great food and the occasional good book.